Supplementary information

FOXO1-miR-506 axis promotes chemosensitivity to temozolomide and suppresses invasiveness in glioblastoma through a feedback loop of FOXO1/miR-506/ETS1/FOXO1

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Materials and methods

1 Quantitative real-time PCR (qRT-PCR) assay

The total RNA of cells was isolated using Trizol reagent or miRNeasy mini kit (invitrogen, USA) in our study. The qRT-PCR reactions were carried out according to the product manual of All-in-One™ qRT-PCR detection kit for miRNA (FulenGen, Guangzhou, China). During the PCR assay in real-time, iQ-5 (Bio-Rad) was used as a monitor. The primer sequences of FOXO1 used were as follows: Forward primer (5'–3') CCGGAGTTTA GCCAGTCCAA and Reverse primer (3'–5') CACGCTCTTGACCATCCACT. The specific primers of miR-506 were purchased from FulenGen Co., Ltd. Cycling profile of PCR was denatured for 5 min at 95 °C, then 40 cycles of annealing for 10 s at 95 °C, and extended for 45 s at 60 °C. For further calculation, the average cycle threshold (C_t) was obtained from three independent experiments. The method of $2^{-\Delta\Delta C_t}$ was used to quantify the relative expression of genes.

2 CCK8 assay

U251 cells (0.5×10^5 well^{-1}) were plated in 96-well plates and cultured for 12 h in 5% CO_2 conditions. TMZ with final concentrations of 0–1500 μmol/L were added to the cells for 48 h. Concentration 0 is that cells treated with solvent as DMSO (0.5%) of TMZ. Cell Counting Kit 8 (Dojindo, Kumamoto, Japan) was used to measure the cell viability according to the instructions.

3 Colonies formation assay

The virus-infected cells were plated in 6-well plates at a density of 5×10^3 cells/well and incubated in culture medium for 1 week with/without treatment with 50 μmol/L TMZ in 0.5% DMSO. The cells were fixed with 70% (v/v) ethanol and stained with 0.5% crystal violet (Genemed, GMS10007) for 0.5 hours at 37 °C. Visible colonies were counted in five different fields, and the mean value was calculated.

4 Western blotting

The total proteins of cells were isolated with a protein extraction kit (KeyGEN BioTECH, China) according to the product manual. Then the proteins were added to 15% SDS-polyacrylamide gels for separation. After that, the blots were transferred to PVDF membranes (Millipore, USA). Then being blocked by 5% defatted milk, the membranes in the company with the primary antibody were incubated overnight. After being washed, the membranes were incubated with the corresponding secondary antibody conjugated by horseradish peroxidase (HRP) which was visualized by a system of enhanced chemiluminescence (ECL) (Pierce Biotechnology, Rockford, IL). Anti-Tubulin (1:1000) (Abcam, UK), anti-LC3B (1:500) (Abcam, UK), anti-Beclin-1 (1:500) (CST, USA), anti-N-Cadherin (1:500) (Abcam, UK) and anti-E-Cadherin (1:500) (Abcam, UK) were used as primary antibodies. Tubulin was used to normalize the results to correct loading.

5 Migration and invasion experiments

Scratch assay experiments were performed to assess cell migration according to a previous study (Chen et al., 2019). Briefly, U251 cells with/without transfection were cultured in 6-well plates for 24 h and then scratched followed by another 48 h incubation. Pictures were taken at 0, 14 and 24 h after scratching via a microscope. Cell migration had been analyzed as the ratio of uncovered area at 48 h to 0 h.

Invasion of cells was performed by trans-well plates (Corning, USA) based on the products' recommendations. A total of 25 μL Matrigel (BD, USA) was added to the trans-well filter insert (Corning, USA) to coat the upper surface of a polycarbonate membrane, and then solidified by incubation at 37 ℃ for 2 h to serve as the extracellular matrix for cell invasion analyses. A total of 1×10^5 cells were collected and re-suspended in 100 μL DMEM medium without serum and then planted into the upper chamber of the plates. 500 μL DMEM containing 10% FBS was added to the lower chambers. After incubation for 24 h, non-migrating cells were scraped to remove from the top of the trans-well membrane. Cells that invaded the bottom of the trans-well membrane were fixed for 20 min by 500 μL 4% paraformaldehyde and then stained by crystal violet. The absorbance values (OD) at 570 nm acquired by a microplate reader were used to quantify the number of migrated cells.

6 Electrophoretic mobility shift assay (EMSA)

EMSA was performed to explore miR-506 promoter binding with FOXO1 protein from U251 cells transfected with FOXO1-OE. EMSA was performed according to the instructions of EMSA/Gel-Shift Kit (Beyotime Biotechnology). In brief, 2 μL of labeled probes (1.75 pmol/μL) was then incubated with 2 μL of nuclear protein of U251 cells with or without transfection of NC/FOXO1-OE. Following the electrophoresis and gel-blotting, the EMSA glue was dried and exposed to x-rays for 20 min at room temperature.

7 Immunofluorescence

After transfection, treated with miR-506 inhibitor and starvation for 12 h, as well as treated with/without 10 ng/mL recombinant ETS1 protein (Abcam, UK), cells on the glass were fixed by 4% paraformaldehyde and then incubated with primary antibody of LC3 (1:200), LAMP2 (1:500) or FOXO1 (Abcam, UK) overnight at 4 °C in the wet box. After being washed with TBST buffer, cells were incubated with secondary antibody and incubated at 37 °C in wet box for 90 min. DAPI Reagent was used to stain nucleus. Cells were observed by fluorescence microscope and photographs were taken.

8 Immunohistochemistry

Tumor tissue from mice was fixed in 4% Paraformaldehyde (PFA) followed by being embedded and sliced. Sections were deparaffinized and hydrated and then incubated with Ki67 antibody overnight at 4 ℃, afterwards incubated with DAB labeled secondary antibody at 25 ℃. After counterstained by hematoxylin, the sections were allowed to take photomicrograph (Olympus, Japan).

Table S1 Primers of miR-506 promoter used in qPCR assay

miR-506 promoter	Primer	Primer sequence (5'–3')	Product (bp)
F1	Forward	CTGAAATGGCGGAGTGAGGG	76
	Reverse	ACACATGCATGGCACTTCTTG	
F2	Forward	CTCCTTCCTACACTCGCAGC	96
	Reverse	TCCCAAACAAAACAGAGAACGAAG	
p1	Forward	ACTGGTTTGCAAGTGCATGA	82
	Reverse	TGGTTTGGCATCCTCTGTTCA	
p2	Forward	GGGTCTGGTGGCACAT	109
	Reverse	GGCAATGGCACAATCT	
p3 or F3	Forward	ACTTCTCAGTGTCCACTGCTC	75
	Reverse	ACGTATCAGGGATAGACGACA	
Negative	Forward	AGCGTCGTTGATACATGACTGA	76
Control	Reverse	AGGGGATGATTTCATGGGTGT	